HALOGENATED HYDROCARBONS AND DRUG METABOLISM: THE EFFECT OF FLUORO-CARBONS ON HEXOBARBITAL SLEEPING AND ZOXAZOLAMINE PARALYSIS TIMES IN MICE

Ethard W. Van Stee, et al

Aerospace Medical Research Laboratory
Wright-Patterson Air Force Base, Ohio

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Ethard W. Van Stee, Major, USAF, VC
James P. F. Murphy
Kenneth C. Back, Ph.D.

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13. ABSTRACT

This report was presented at the Proceedings of the 2nd Annual Conference on Environmental Toxicology, sponsored by the SysteMed Corporation and held in Fairborn, Ohio on 31 August, 1 and 2 September 1971. Major technical areas discussed included toxicological evaluation of volatile halogenated compounds, protection of the public against air pollution and toxicological problems with aircraft, missiles, and space vehicles.

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Toxicological screening
Gas chromatography
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PAPER NO. 6

HALOGENATED HYDROCARBONS AND DRUG METABOLISM: THE EFFECT OF FLUOROCARBONS ON HEXOBARBITAL SLEEPING AND ZOXAZOLAMINE PARALYSIS TIMES IN MICE

Ethard W. Van Stee, Major, USAF, VC
James P. F. Murphy
and
Kenneth C. Back, Ph. D.

Aerospace Medical Research Laboratory
Wright-Patterson Air Force Base, Ohio

A screening program has been initiated for the purpose of exploring the possibility that the inhalation exposure of rodents to selected halogenated hydrocarbons may affect enzymes of the hepatic smooth endoplasmic reticulum, the so-called microsomal drug-metabolizing enzymes. The materials of interest to the Air Force which have come under investigation are shown in figure 1 and include bromotrifluoromethane, trichlorotrifluoroethane, and dibromotetrafluoroethane. Some work with halothane has also been done for comparative purposes.

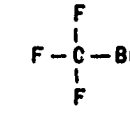
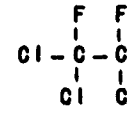
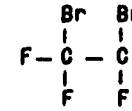
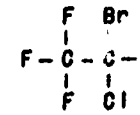
STRUCTURAL FORMULA	NOMENCLATURE	COMMENTS
	BROMOTRIFLUORO-METHANE FREON 1301 F1301, F13B1	B.P.-57.8 C USES: FIRE EXTINGUISHING AGENT PROPELLANT
	1,1,2-TRICHLORO-1,2,2-TRIFLUORO-ETHANE FREON 113, F113 FREON TF SOLVENT FREON PRECISION CLEANING AGENT	B.P.+47.6 C USES: SOLVENT AND DEGREASER REFRIGERANT
	1,2-DIBROMO-1,1,2,2-TETRAFLUORO-ETHANE FREON 114B2, F114B2 HALON 2402, H2402	B.P.+47.3 C USE: FIRE EXTINGUISHING AGENT
	1-BROMO-1-CHLORO-2,2,2-TRIFLUORO-ETHANE HALOTHANE	B.P.+50.2 C USE: GENERAL ANESTHETIC

Figure 1.

Begin - 71

$CBrF_3$ is a fluorocarbon which boils at about -58 C. It is a gas under standard conditions that is stored as a liquid under pressure. $CBrF_3$ is an effective fire-extinguishing agent that, although pharmacologically quite active, is tolerated by most species in comparatively high concentration by inhalation exposure. The most likely routes of exposure would be pulmonary or cutaneous. The gas is readily absorbed following inhalation and would be expected to be absorbed percutaneously to a certain extent because of its solubility in nonpolar materials. The discharge of $CBrF_3$ in the vicinity of a fire would be expected to produce rather high local concentrations of the material for brief periods in terrestrial environments. In extraterrestrial environments, longer potential exposure periods might be expected because of the problems involved in the purging and recharging of the life support system atmosphere.

Trichlorotrifluoroethane boils at about 48 C and is thus a liquid under standard conditions. Because of its relatively high vapor pressure under the usual conditions of its use, the material evaporates readily and relatively high concentrations of the gas phase could be expected in the vicinity when it is being used in its primary role as a solvent and degreaser. Brief cutaneous exposures to the liquid and pulmonary and cutaneous exposures to the gas of varying durations might be anticipated in practice.

H2402 is a candidate fire-extinguishing agent with physical properties similar to those of F113. The primary application by the USAF is expected to be in hand-held fire extinguishers for use aboard aircraft and in other closed and semi-closed environments. Because of the nature of its use, brief exposures to liquid and gas might be anticipated during its application to a fire. Since systems using this agent are now in the developmental phase, the possibility of repeated short-term or even chronic exposures exists for the personnel engaged in design and testing.

The anesthetic agent halothane bears a structural relationship to the other fluorocarbons mentioned and it is not surprising that it also shares some pharmacological-toxicological properties. Since its introduction in 1956, an enormous amount of research on its properties has been performed throughout the world. Because so much is known about halothane and because of its similarities to the fluorocarbons of interest to the Air Force, we have, in our laboratory, often included it in our studies for comparative purposes.

A rational approach to the evaluation of the effect of a drug or chemical on drug metabolism in an organism requires that a logical sequence of steps be performed as shown in figure 2. This process has only begun in our laboratory in our study of fluorocarbons, but we intend to pursue this line of study as long as we continue to remain reasonably confident of fruitful results. Our general approach to the problem of the effect of fluorocarbons on hepatic microsomal enzymes has been that which was outlined by Fouts (1971) in Methods in Pharmacology, edited by Schwartz. The first step in the program was the determination of the effects of exposure of male mice to the respective fluorocarbons on the duration of hexobarbital sleep and zoxazolamine

paralysis. The pharmacological basis of this test is the assumption that the duration of sleep or paralysis is largely an inverse function of the rate of biochemical inactivation of the barbiturate or muscle relaxant as shown in figures 3 and 4. In these cases, the conversion of most of the hexobarbital to 2 isomers of the oxidation product, ketohexobarbital, or the conversion of zoxazolamine to 6-OH-zoxazolamine is known to be mediated by enzymes of the hepatic smooth endoplasmic reticulum. The activity of these enzymes has been observed to be increased in a large number of species of animals following their exposure to a wide variety of organic compounds. By increasing the activity of the drug-metabolizing enzymes, the rate of inactivation of the compounds is increased and the duration of their pharmacologic effects decreased (figure 5). This test for induction is quite imprecise and is used only as a screening tool for selecting agents for further study which might be expected to elicit a marked alteration of enzyme activity in the test animals.

Once the determination has been made that exposure to the agent in question significantly alters the sleep and/or paralysis times, the next step in the investigation is undertaken (figure 6). Although the most likely reason that exposure to a fluorocarbon leads to a change in sleep or paralysis time is that the fluorocarbon led to a change in the rate of metabolic degradation of the hexobarbital or zoxazolamine, it is possible that a change in the agonist-receptor interaction could be induced which could in turn result in an altered sensitivity of the organism to any given concentration or dose of hexobarbital or zoxazolamine. In order to either eliminate from consideration or identify this phenomenon for further investigation, control and induced animals are given the standard doses of hexobarbital or zoxazolamine. The animals are killed by cervical dislocation at the appropriate pharmacological endpoint and the brain levels of the respective materials determined. If there is no significant difference in the waking brain levels of the hexobarbital or zoxazolamine between the control and treated groups, it may be safely assumed that no alteration in the animals' sensitivity to these drugs occurred, thereby strengthening the case for the alteration of the rate of metabolic degradation as the principal cause of the alteration of the sleep or paralysis time.

STEPS IN THE DETERMINATION OF THE EFFECTS OF XENOBIOTICS ON THE ENZYMES OF THE HEPATIC SMOOTH ENDOPLASMIC RETICULUM

A. IN VIVO STUDIES

1. HEXOBARBITAL SLEEPING AND ZOXAZOLAMINE PARALYSIS TIMES.
2. WAKING BRAIN LEVELS OF HEXOBARBITAL OR ZOXAZOLAMINE.
3. DECLINE OF WHOLE BODY CONCENTRATIONS OF HEXOBARBITAL OR ZOXAZOLAMINE.

B. IN VITRO STUDIES - RATE OF SUBSTRATE DISAPPEARANCE OR PRODUCT FORMATION

1. LIVER HOMOGENATES.
2. LIVER SUBFRACTIONS.

Figure 2.

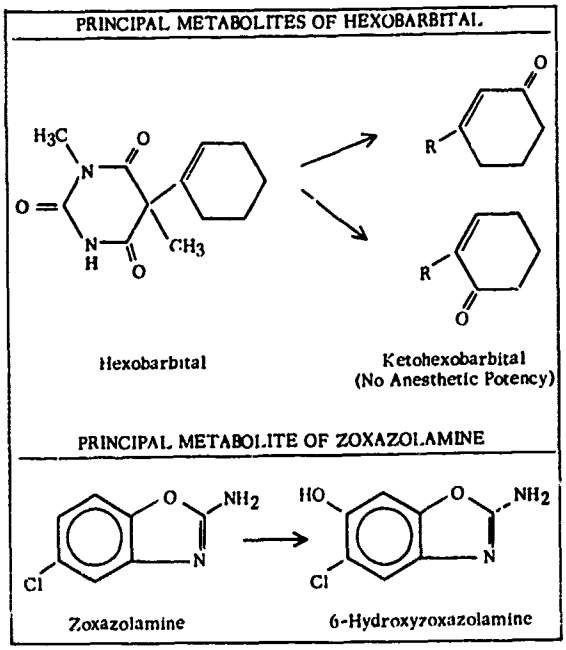


Figure 3.

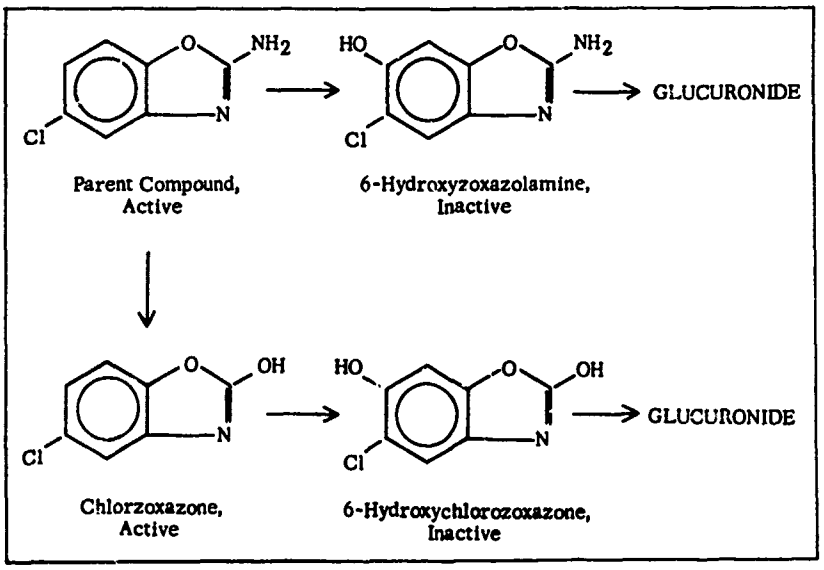


Figure 4. METABOLISM OF ZOXAZOLAMINE

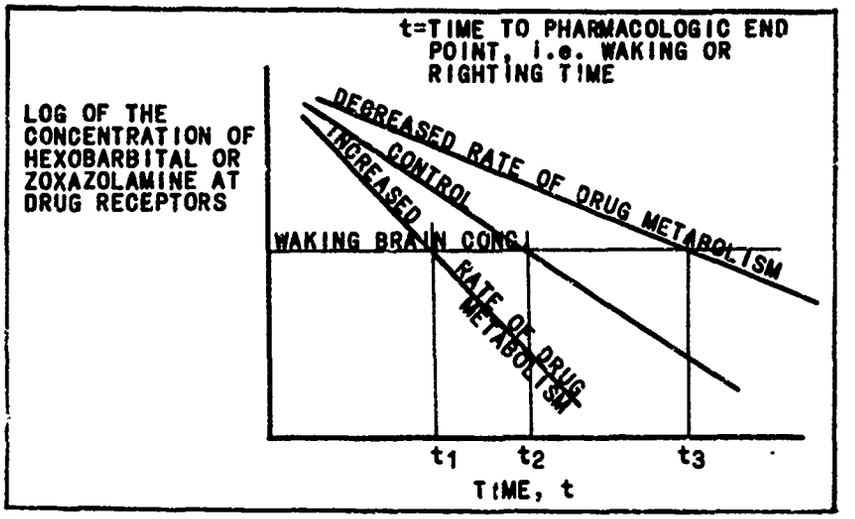


Figure 5. THE EFFECT OF CHANGING THE RATE OF DRUG METABOLISM ON PHARMACOLOGICAL ENDPOINT AND ITS RELATIONSHIP TO BRAIN LEVEL

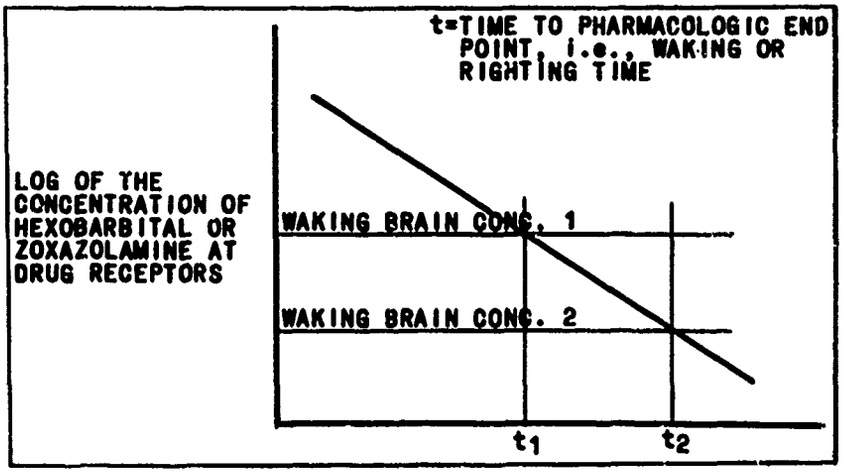


Figure 6. THE EFFECT OF CHANGING AN ORGANISM'S SENSITIVITY TO A DRUG ON PHARMACOLOGICAL ENDPOINT AND ITS RELATIONSHIP TO BRAIN LEVEL

The next step (figure 7) in the analysis of the effect of exposure to fluorocarbons on the hepatic microsomal enzyme system is the final in vivo step. The rate of decline of whole body levels of hexobarbital, zoxazolamine, or other test material (commonly compounds such as antipyrine, aminopyrine, ethylmorphine etc.) is determined as a reasonable estimate of the rate of metabolic degradation of the compound. Groups of test animals, control or induced, are injected with the drug whose rate of metabolism is to be followed. The animals are killed serially in groups of 3-5 and the whole body concentrations of the parent compound determined. These values may be plotted versus time and the time required for the whole body concentration to be reduced by one-half calculated. This is the so-called biological half-time and represents a commonly determined index of the rate of metabolic degradation of xenobiotics.

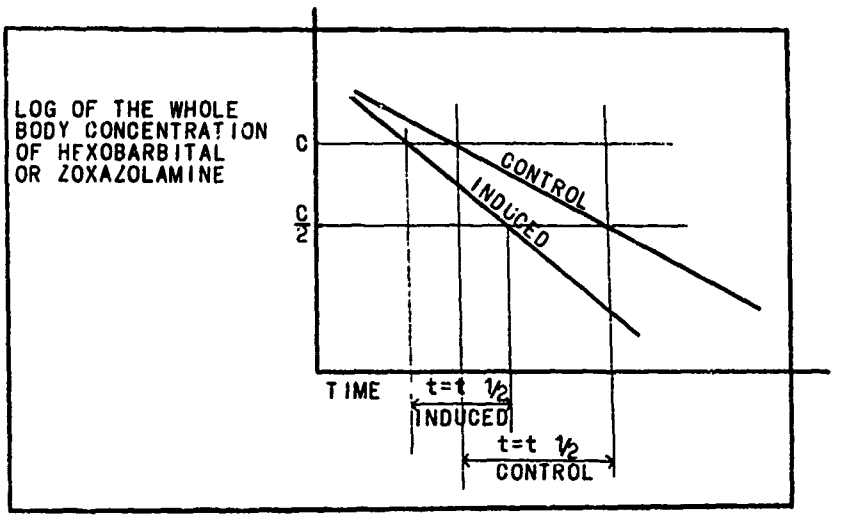


Figure 7. DETERMINATION OF THE TIME REQUIRED FOR THE WHOLE BODY CONCENTRATION OF HEXOBARBITAL OR ZOXAZOLAMINE TO DECLINE BY ONE-HALF, $t_{\frac{1}{2}}$.

In vitro studies logically follow the in vivo studies (figure 2). The rate of disappearance of substrate in homogenates and various liver subfractions may be used to characterize more precisely the nature of the changes induced in the hepatic smooth endoplasmic reticulum. In our studies of fluorocarbons, we are still weeks from the in vitro work and we will not consider it in any more detail at this time.

Dr. E. S. Vesell (1968) of the NIH published a report in 1968 which discussed factors altering the responsiveness of mice to hexobarbital. If I may be permitted to quote from his paper, "The pharmacologic responsiveness of mice to hexobarbital is altered by both genetic and environmental factors including age, sex, litter, strain, bedding, painful stimuli, ambient temperature, grouping, and hour of administration." These factors were borne in mind in the course of the determination of the sleep and paralysis times. Male mice were obtained from the same supplier which we hope has resulted in some degree of strain uniformity. All mice were 28 ± 2 days old on the first day of exposure Environmental factors were kept as nearly constant as possible and all necessary interventions performed as nearly uniformly as possible. The same operator performed all of the sleep studies and his subjectively-determined criteria for time of loss and time of regaining righting capability were applied uniformly to all animals.

To the list of reasons for differences in responsiveness to hexobarbital we may add variations of the mechanics of injection and the properties of the hexobarbital and zoxazolamine solutions (figure 8). The zoxazolamine was prepared for injection by first dissolving it in polyethylene glycol. The hexobarbital was dissolved in physiological saline adjusted to a high pH with sodium hydroxide. The final solutions were then injected intraperitoneally on the basis of weight. Injections were made with a 25 gage needle into the lower right quadrant. Occasionally, a mouse would not lose its righting capability at all. This can result from the injection of the bolus into the lumen of the intestine or bladder. Occasionally, others would die in a few minutes or sleep for exceedingly long times with or without ultimate survival. Because the mouse response can be so variable, it is necessary when evaluating sleep and righting times to establish criteria for rejecting outliers (figure 9). To begin with, all mice that failed to go down at all and those that slept for inordinate periods were eliminated. The remaining values for sleeping or paralysis time which lay beyond the mean plus or minus two times the standard deviation were eliminated from the group of animals which remained down from one to 400 minutes. The final means, standard deviations, and n-values are from the samples corrected according to these criteria.

DRUG	PREPARATION	INJECTION
ZOXAZOLAMINE	INITIALLY DISSOLVED IN PEG 200 - THEN DILUTED 1:1 WITH DISTILLED WATER FINAL CONCENTRATION: 10 mg/ml	100 mg/kg IP
HEXOBARBITAL	DISSOLVED IN NaOH SOLN, TITRATED BACK TO pH 9-10 WITH HCl, DILUTED TO FINAL CONCENTRATION OF 12 mg/ml WITH 0.9% NaCl	120 mg/kg IP

Figure 8.

SLEEP TIME EXCLUSION CRITERIA

1. EXCLUDE 0-MIN., > 400 MIN
2. REMAINDER: $0 < X < 400$ MIN
   CALCULATE MEAN, STD DEV
3. EXCLUDE VALUES WHICH LIE OUTSIDE
   $X \pm 2.0$ S.D.
4. RECALCULATE FROM CORRECTED SAMPLE:
   MEAN, STD DEV, N

Figure 9.

The next six figures illustrate the results of the determination of hexobarbital sleeping and zoxazolamine paralysis times in mice treated with four different fluorocarbons. All of the exposures were carried out on groups of 30 mice in a plexiglas exposure chamber of our design. The concentrations of fluorocarbons were checked as necessary using a gas chromatograph. All exposures were for 5 hours and were repeated for three consecutive days. The hexobarbital and zoxazolamine times were determined 24 hours following the third and final exposure session.

As illustrated in figure 10, exposure to 80% $CBrF_3$ had no effect on either hexobarbital sleeping or zoxazolamine paralysis time. Further exposures to $CBrF_3$ have not been performed in this study because 80% represents an extremely high concentration. It is understood that some compounds are known that have a delayed effect on the drug-metabolizing enzymes and that any compound cannot be classified as not an inducer with any degree of certainty until animals have been continuously exposed for 3 weeks or so without altering the sleep times. Nevertheless, our initial screening program has been limited to 3-day intermittent exposures for the purpose of detecting possible potent inducers which might be expected to produce an early effect.

The next two figures (11, 12) illustrate the results of the exposure of mice to F113. Exposure to 2% did not result in a significant alteration of the sleep times; however, exposure to 3% resulted in a significant decrease in the duration of both the hexobarbital and zoxazolamine times.

Exposure to 0.75% halothane yielded inconclusive results (figure 13). Although the hexobarbital sleeping times yielded a significant value of "t" at the 95% level, zoxazolamine paralysis time was unaffected. Repeating the experiment with 1% halothane did

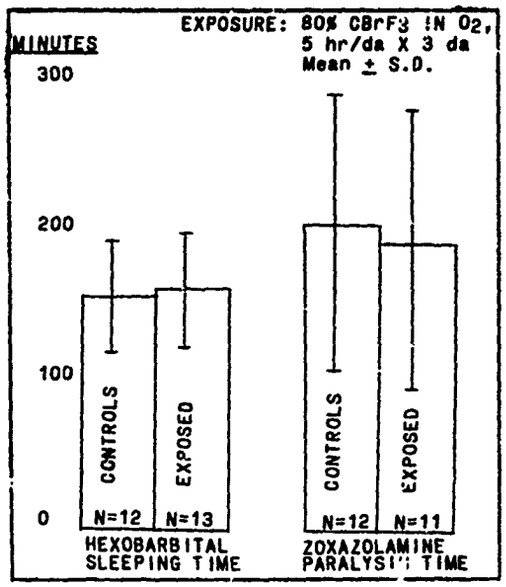


Figure 10.

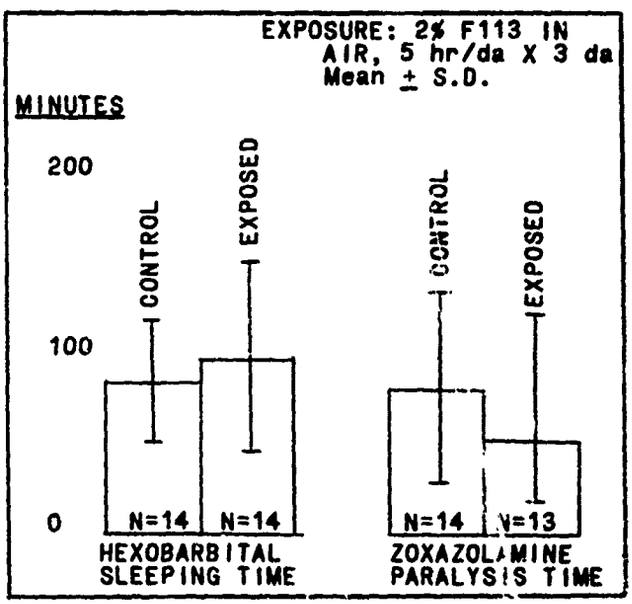


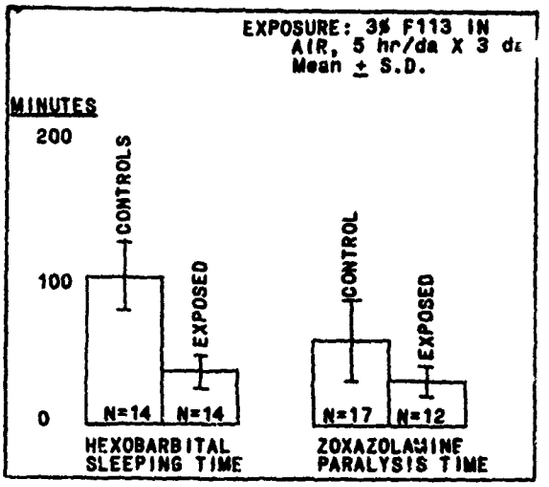
Figure 11.

not result in a significant alteration in either time (figure 14). Other workers have had similar experience with halothane and the only consistency seems to be the equivocal nature of the results.

Soon after beginning an evaluation of H2402 it became apparent that this compound was a likely canidate as a potent inducer of drug-metabolizing enzymes. Several experiments were performed in which groups of mice were exposed to concentrations from .25% to 2.5% with the results which are illustrated in figure 15. The duration of hexobarbital sleeping times and zoxazolamine paralysis times are expressed as percentage of control in order to pack as much information as possible into figure 15. It may be seen that the duration of both times follow a similar course. They decline from 0.25% and reach a plateau which extends from about 0.5% through 1.0%. Above 1.0% they begin to rise again. The statistical significance can be appreciated if an imaginary line parallel to the abscissa is drawn at about 75% of control. All values below that line represented statistically significant differences using Student's t-test.

Based on the results of the sleep studies which have been described we have concluded that any effect which $CBrF_3$ might have on the metabolizing enzymes of the hepatic smooth endoplasmic reticulum would be likely to be minimal within the context of its application as a fire-extinguishing agent. Our opinion is that the evidence does not warrant our further consideration of it at this time as a possibly significant enzyme inducer.

Freon® 113 and 114B2, in contrast to 1301, may be suspected to possess the potential to alter the microsomal enzyme systems. The evidence is not as clear-cut in the case of 113 as it appears to be with 114B2. In both cases, however, the evidence would seem to be substantial enough to justify proceeding to the next in vivo steps which are listed in figure 2; namely, determination of the waking brain levels and whole-body half-life of hexobarbital and/or zoxzolamine.



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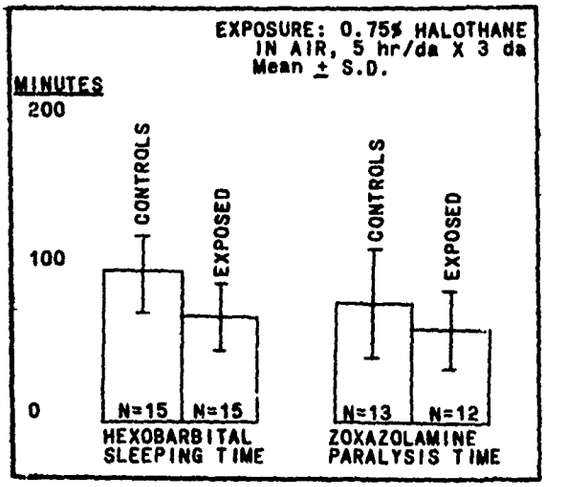

Figure 13.

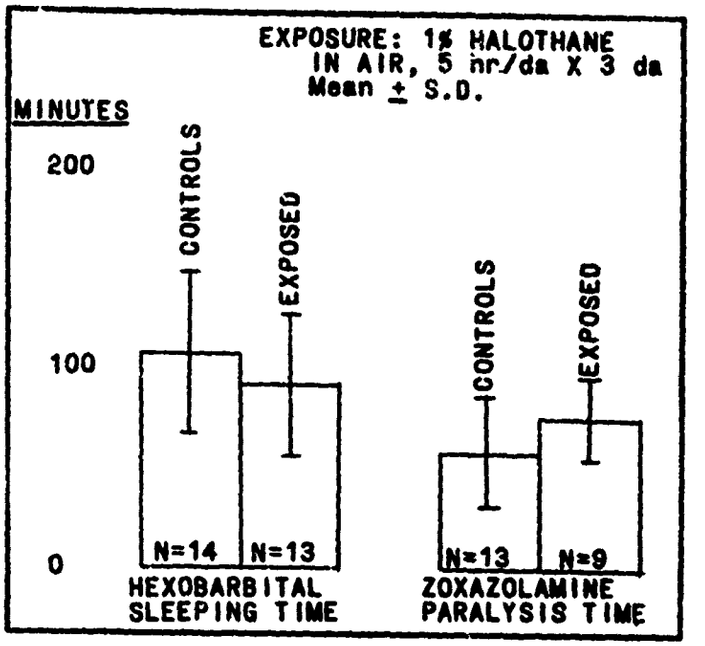

Figure 14.

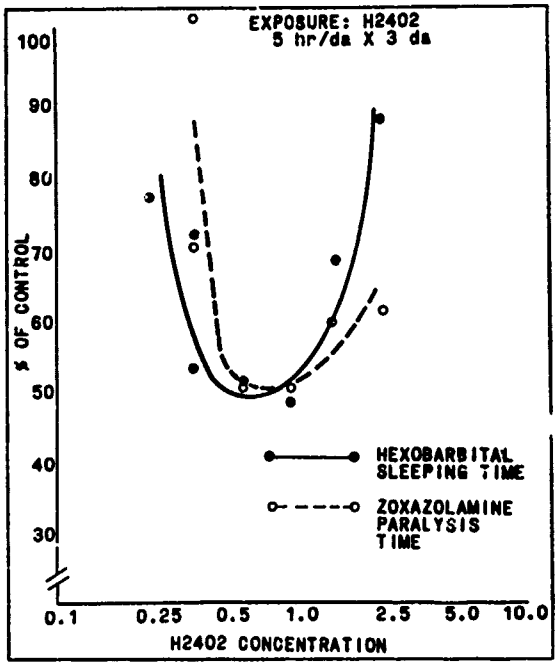

Figure 15.

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DISCUSSION

DR. CALDERWOOD (University of Florida): I just want to bring to your attention a paper recently published in the Journal of Anesthesiology by Sawyer, who is also a veterinarian, working with the metabolism of halothane in miniature pigs. If memory serves me correctly, part of the conclusion was that at very low levels of halothane in the blood, the amount of metabolizing enzyme produced by the liver was decreased. I suggest you read the paper and get it more accurately than I can remember it, but it might have some application here in other areas, in that with the low level of halothane decreasing the rate of metabolism of halothane, you get a prolonged period of halothane in the blood.

MAJOR VAN STEE (Aerospace Medical Research Laboratory): Don Sawyer is a friend of mine and I should have all of his papers. I think I may have it back in my office, but honestly I don't recall specifics at the moment.

DR. SMITH (Federal Aviation Administration): On exposure to the inducing agent itself, it did have a profound pharmacological or toxicological effect?

MAJOR VAN STEE: If you are referring to the behavioral effects on the mice in the exposure chamber, yes, there most certainly were, and these were most obviously dose-dependent because they ranged from no response to death and we had various dearrangement of locomotion. This began at some of the lower concentrations and progressed as we approached lethal concentrations at about 5%. Of course, there was lethargy and inactivity at the higher concentrations also.

LT. COL. STEINBERG (Army Environmental Hygiene Agency): Your results help explain what we thought were some screwy results because about three years ago, we were looking at Freon 113 and its use in a solvent system. We took levels up to 5000 ppm for thirty days, and up to six weeks, and found that in running tests as the concentration increased the running would decrease (this is an activity measurement). We did a battery of chemistries and were unable to demonstrate any hepatotoxicity at all. Fortunately, the problem was dissipated before we had to pursue it.

DR. BACK (Aerospace Medical Research Laboratory): One of the things that Dr. Smith was wondering about was that these animals actually convulsed in lower dose ranges. I don't know if you call it a convulsion or not, anyway they pop like popcorn. This was at about 2%. They do pop like popcorn and then they become lethargic after the initial induction of whatever you want to call it - whether they're convulsions or not. Of course, I would guess that we had marked adrenal discharge, but we never lost any animals under these conditions - showing one thing, that endogenous epinephrine was not sufficient to kill the animals during the convulsive state.

MAJOR VAN STEE: I'd like to make one brief comment on that. We must consider the species of animal involved here, if we're making even indirect references to cardiac arrhythmias, particularly ventricular fibrillation. It is reasonably well known that very small hearts are most difficult to fibrillate, and I don't know that it is possible to fibrillate a mouse heart and thereby cause death. I do know that it is very difficult to cause an irreversible ventricular fibrillation in an animal such as a monkey, whereas in a dog it is very easy to bring about. Perhaps other people here would care to comment on that, but I doubt that cardiac arrest following ventricular fibrillation would be significant in the mouse.